CLXXIII. OBSERVATIONS ON THE FUNCTION OF PEROXIDASE SYSTEMS AND THE CHEMISTRY OF THE ADRENAL CORTEX.

DESCRIPTION OF A NEW CARBOHYDRATE DERIVATIVE.

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Introduction.

Eight years ago observations were made on the adrenalectomised animal which gave the impression that the adrenal cortex is in some way involved in the mechanism of biological oxidation. A detailed study of the biological oxidation was begun in the hope that this study might lead to the understanding of the function of the interrenal system.

Through several years different animal, vegetable and synthetic oxidising systems were studied [Szent-Györgyi, 1924, 1, 2; 1925, 1, 2; 1927, 1, 2], but no connection could be found between these and the function of the adrenal cortex.

Finally the peroxidase system was examined. Evidence is presented in the present paper that the adrenal cortex is connected in some way with this oxidising mechanism.

PART I.

On the function of peroxidase in the plant.

1. *Some observations on the guaiacum reaction.*

If to the solution of a purified peroxidase a few drops of an alcoholic solution of guaiacum and a few drops of 0·01 *N* hydrogen peroxide are added, the immediate development of a blue-green colour proves the activity of the peroxidase. But if the same reagents are applied to the press juice of the plant, which contains almost the whole of its peroxidase, the reaction remains negative, and no colour occurs. This negative result has hitherto generally been attributed to the action of inhibitory substances.

If, for instance, from the roots of the turnip (*Brassica rapa*) a press juice is made, and the above reagents added, no colour appears, although the fluid contains a very active peroxidase which, if reprecipitated with acetone, gives at once a very strong reaction.

If however the quantity of the peroxide added to the crude juice is gradually increased, after a certain quantity of peroxide has been added the green colour suddenly appears and becomes rapidly deeper on the further addition of peroxide. The appearance of this colour depends on the quantity of peroxide added. If a quantity of peroxide is added just insufficient to cause the colour, standing for any length of time does not bring the colour out. Thus the crude juice behaves in the same way as the purified peroxidase, but only after the addition of a certain quantity of peroxide.

This behaviour indicates that the retardation of the reaction in the crude juice is not due to an inhibition in the real sense, but that the first quantities of H_2O_2 added are not able to oxidise the guaiacum, because they are used up in another reaction[1].

The presence of strongly reducing substances in the juice is also indicated by the rapid reduction of iodine. If to the juice starch and then 0·01 *N* iodine are added, the first quantities of the iodine are rapidly reduced. This allows the titration of the juice with fair accuracy with 0·01 *N* iodine. (The end-point of the titration is reached when the reduction of iodine becomes suddenly very slow.)

If, however, peroxide is added before the iodine, the iodine reduction will be found smaller; the decrease of the iodine uptake is equivalent to the peroxide added. The substances which reduce iodine are thus oxidised by the peroxide.

This experiment shows that the first quantities of peroxide, which are unable to colour guaiacum in the crude juice, are used up for the oxidation of a substance which can be oxidised equally well by iodine.

This can clearly be demonstrated if the experiment is made in the reverse order and the iodine is added first, and is followed by the peroxide. In this case the quantity of H_2O_2 required for the coloration of guaiacum is diminished by an amount equivalent to the iodine previously added. If sufficient iodine has been added, the first drop of H_2O_2 will cause the blueing of the guaiacum. Thus, if the reducing substance is oxidised by iodine, it is unable to use up H_2O_2.

This behaviour of the crude juice may be illustrated by the following experiment.

Exp. The root of the turnip is cut up and minced. The juice is pressed out through muslin in a hand press and freed from suspended particles in the centrifuge. The final product is a milky fluid. During the experiment this is kept in large test-tubes so as to reduce its contact with air.

To 5 cc. of the juice a few drops of alcoholic guaiacum solution are added. Then, drop by drop, 0·01 *N* H_2O_2 is run in, the fluid being mixed after the addition of every drop. No colour is seen on the addition of the first 1·5 cc. nor does it appear on standing. After addition of 1·6 cc. a green colour appears and becomes rapidly deeper on further addition of H_2O_2.

[1] Catalase could not account for the disappearance of H_2O_2 since the juice shows only a very feeble catalase activity.

To 5 cc. of the above juice 2 drops of strong HCl are added (to inactivate the peroxidase). Then the fluid is titrated with iodine in presence of starch. Uptake 1·9 cc. of 0·01 *N* iodine.

To 5 cc. of the same juice 1·9 cc. of 0·01 *N* H_2O_2 are added, the fluid is mixed and after a few seconds acidified with HCl. On titration 0·4 cc. of 0·01 *N* iodine is taken up.

Thus after the addition of H_2O_2 1·5 cc. less iodine is used.

To 5 cc. of the same juice 1·9 cc. of 0·01 *N* iodine and then a few drops of guaiacum tincture are added. On addition of the first drops of H_2O_2 the guaiacum reaction becomes positive at once.

These experiments thus make it clear that there is in the crude juice a substance (or a set of such substances) which rapidly reduces H_2O_2, and that the first quantities of H_2O_2 added are unable to cause the coloration of guaiacum, because they have been used up for the oxidation of this substance.

This substance, which reduces H_2O_2 and iodine, also reduces phosphotungstic acid and neutral silver. If to the juice 20 % phosphotungstic acid and then a few drops of ammonia are added, a deep blue coloration shows the reduction of the acid[1]. If to the neutralised juice silver nitrate is added, this is rapidly reduced. Both reductions are absent if H_2O_2 has been added previously.

This constituent of the crude juice which reduces the H_2O_2 and can be also detected by the reduction of iodine, silver or phosphotungstic acid will be referred to in the following pages as the "reducing factor" (R.F.).

2. *Mechanism, rate, nature and significance of the oxidation of the* R.F. *and its distribution.*

Mechanism. The first question which arises is whether the oxidation of the R.F. by H_2O_2 is a direct oxidation, or an oxidation catalysed by the peroxidase. This question can easily be answered by the measurement of the rate of the oxidation in presence and absence of peroxidase. As the following experiment shows, the rate of oxidation of the R.F. by H_2O_2 is greatly reduced if the enzyme is inactivated by heating. A similar result is obtained if the enzyme is inactivated by cyanide, so that we can safely conclude that the rapid oxidation of the R.F. by H_2O_2 is a peroxidase oxidation.

Exp. 5 cc. juice were titrated with 0·01 *N* iodine as in Exp. 1. 5 cc. of the same juice were titrated in the same manner after the addition of 2·0 cc. 0·01 *N* H_2O_2. Iodine uptake in the first = 2·0 cc., in the second = 0·5 cc.

The same experiment was repeated with juice which had previously been rapidly heated to boiling and cooled. Iodine uptake without peroxide = 2·0 cc., with peroxide = 1·9 cc.[2]

[1] Not all phosphotungstic acid preparations could be used for this test. Some preparations gave only very weak colours. Yellow-coloured preparations seemed to give better reactions, but it is not certain whether there is any relation between the yellow colour and the reaction. It is not impossible that the reaction is dependent on some molybdenum compound present as impurity, since the R.F. also strongly reduces phosphomolybdic acid to a highly coloured deep blue product. The phosphotungstic reaction itself is not sensitive to H_2O_2.

[2] If to the boiled juice peroxidase is added, the R.F. is again readily oxidised by H_2O_2.

If the boiled juice is allowed to stand with the peroxide for 5 minutes, and titrated only after this time with iodine, it still takes up 1·0 cc. of the latter.

Rate. The most suitable method for following the rate of oxidation of the R.F. is given by the phosphotungstic acid reaction. If a small quantity of juice is added to a solution of phosphotungstic acid, and then a few drops of ammonia added, the developing blue colour indicates the presence and quantity of the unoxidised R.F. This same experiment can be repeated at short intervals after the addition of the H_2O_2.[1] This experiment shows that the oxidation of the R.F. by the H_2O_2 is extremely fast. Within 5 seconds the whole factor is quantitatively oxidised, even if the peroxide is added but in slight excess. I was in fact unable to take out the sample quickly enough after the addition of H_2O_2 to detect unoxidised reducing substance.

Exp. 1 cc. of 20 % phosphotungstic acid is pipetted into each of a series of tubes.

2·5 cc. of turnip juice are titrated with 0·01 *N* iodine. Uptake 0·7 cc.

2·7 cc. of the same juice are put in a test-tube and 0·7 cc. 0·02 *N* H_2O_2 are added. Before and immediately after the addition of the H_2O_2 a sample of 0·2 cc. is taken and placed in a tube containing phosphotungstic acid.

Then to each tube containing phosphotungstic acid a few drops of strong ammonia are added. On addition of ammonia the first fluid turns deep blue, the second remains colourless.

The next question regards the *nature* of the oxidation of the reducing factor. Is it an irreversible oxidation corresponding to the final oxidation of an intermediary metabolic product, or is it a reversible oxidation corresponding to the oxidation of a catalytic hydrogen carrier?

To decide whether the oxidation is a reversible one, the substance was oxidised by peroxide and peroxidase, and then treated with a mild reducing agent (H_2S). This experiment clearly showed that the oxidised R.F. can be quantitatively reduced again. The oxidation of the R.F. by H_2O_2 and peroxidase is therefore a reversible one.

Exp. To 15 cc. of turnip juice 9·3 cc. of 0·01 *N* H_2O_2 were added. The negative phosphotungstic acid reaction showed that the R.F. had been oxidised completely. To a second and equal sample 9·3 cc. water were added. The fluid showed the usual strong phosphotungstic acid reaction. Through both fluids a slow stream of H_2S was led for half an hour. Then to both fluids four drops of pure formic acid were added. The fluids were poured into wide beakers. In another beaker was put a strong solution of lead acetate. The beakers were placed over sulphuric acid in the desiccator which was then evacuated. Next morning the volume of both fluids was found to be 15 cc. Both showed by a negative nitroprusside reaction that the H_2S had been removed completely. Both fluids showed an equally strong phosphotungstic acid reaction. To 9 cc of each fluid 2 cc. of a strong solution of purified peroxidase were added. From each of these two fluids two samples of 5 cc. were taken. One pair of samples

[1] The peroxidase is at once inactivated by the phosphotungstic acid and any further oxidation is stopped.

was titrated with 0·01 *N* iodine (in presence of HCl). Both showed an iodine uptake of 2·3 cc. To the other pair 2·3 cc. 0·01 *N* H_2O_2 were added, and the fluids titrated with iodine as above. Iodine uptake in both fluids was 0·3 cc.

When it had been shown that the oxidised R.F. could be easily reduced by chemical means, the question arose whether natural oxidising systems were also capable of effecting this reduction. To answer this question the R.F. was oxidised, and the juice incubated.

This experiment showed that reduced R.F. reappears in the juice on standing and that this is due to enzymic activity, since it does not happen in the tube in which the enzymes are destroyed by heating.

Exp. 25 cc. of juice were introduced into a Thunberg tube. Then slowly with steady shaking the calculated quantity of 0·01 *N* H_2O_2 was added (7·5 cc.), which was just sufficient to oxidise the whole amount of R.F. present[1]. The phosphotungstic acid reaction of a sample was negative, or showed only a very slight colour, proving that practically the whole R.F. was oxidised. One part of the fluid was heated in a tube to boiling in order to destroy all enzymic activity, and then rapidly cooled. After boiling the fluid showed a faint phosphotungstic acid reaction. Both portions were put in a Thunberg tube. To both samples toluene was added and both tubes were evacuated and closed. After standing for 4 hours at room temperature the tubes were opened, and the phosphotungstic acid reaction performed. The unboiled fluid showed a strong reaction, the boiled sample showed the same faint colour as immediately after boiling.

Significance. It has been shown that the first relatively large quantities of H_2O_2 added to the juice of the turnip are used up for the oxidation of the so-called reducing factor, and it has been shown that the oxidation of this factor is a reversible one, and that under the given conditions this factor plays the rôle of a catalytic hydrogen carrier between the peroxidase and other oxidising, or reducing, systems. It has also been shown that the rate of the oxidation of this factor is extremely high.

If these experiments can be applied to the intact plant, then the current views on the action of the peroxidase will have to be modified. It is generally assumed that the peroxidase, if supplied by peroxide *in vivo*, would oxidise the most different metabolic products. But, as is known from *in vitro* experiments, the rate of the oxidation of such metabolic products, as for instance lactic acid by peroxide and peroxidase is extremely slow compared to the rate of the oxidation of the above reducing factor. Thus while this factor is present no peroxide will be available for the oxidation of metabolic products, and the only cell constituent oxidised by the peroxidase will be the R.F. which, if oxidised, is reduced again by other systems. The peroxidase will thus have to be looked upon not as an enzyme which oxidises the different metabolic products directly, but as an enzyme having a specific substrate, which,

[1] It is necessary to avoid any excess of H_2O_2 because, as Dixon has shown, H_2O_2 has a very strong inactivating action on certain oxidising enzymes.

playing the rôle of a catalytic hydrogen carrier, connects it up with other oxidising, or reducing, systems.

Distribution. The few plants examined in the present research gave the impression that the reducing factor is in its distribution closely connected with the peroxidase. This holds naturally only for the healthy tissue. In ripening fruits, the tissues of which are senescent and colliquating, the proportionality is disturbed through the disappearance of the peroxidase. A strongly developed R.F. has only been found in the so-called "peroxidase plants." The following observations may be noted[1].

The Spanish black radish (*Rafanus sat. niger*) behaved in the same way as the turnip. Both have a highly active peroxidase, and a relatively highly developed R.F.

The leek (*Allium porrum*) has a still higher peroxidase activity than the turnip. The concentration of the R.F. is also distinctly higher.

The Spanish onion (*Allium cepa*) showed only a very low peroxidase activity and a correspondingly low concentration of the R.F.

In the ripe fruit of the pineapple (*Ananas sativus*) there is a fair concentration of R.F. corresponding to that of the turnip, but the peroxidase is considerably weaker. In the ripe fruit of the tomato (*Solanum lycopersicum*) there is about the same concentration of R.F. as in the turnip or pineapple, but the peroxidase is very weak. Both plants are "peroxidase plants" [Onslow, 1921].

The ripe orange (*Citrus aurantium* Risso) has a high concentration of R.F., about twice that of the turnip. It does not contain any active peroxidase.

The lemon (*Citrus lemonum*) and grape fruit (*Citrus decumana*) have a still higher concentration of R.F. than the orange. Orange and lemon are also typical "peroxidase plants."

Cabbage (*Brassica oleracea*) leaves have also a very high concentration of R.F.; 10 cc. of the press juice correspond to 10 cc. 0·01 *N* iodine. The peroxidase activity could not be estimated, since sufficient quantity of juice could only be pressed out after the leaves had been exposed to water vapour.

In oxidase plants the R.F., if present at all, seems to be but very weakly developed. 10 cc. of the fresh press juice of the apple reduce 0·3 cc. 0·01 *N* iodine, while that of the potato reduces 0·9 cc. The corresponding reduction values of the typical peroxidase plants vary in the above experiments between 6 and 10 cc.

[1] The peroxidase activity was examined in the dilute press juice by the guaiacum reaction (in presence of excess of peroxide), and by the rate of oxidation of pyrogallol.

The R.F. was estimated in the press juice by the reduction of iodine, the phosphotungstic acid, reaction and the reduction of neutral silver. In all cases it was also shown that the substance giving these reductions was oxidised at a high rate by peroxide and peroxidase, *i.e.* that after the addition of peroxide, both reactions became rapidly negative. To the juice of ripening fruits, which contained little or no peroxidase, this enzyme was added.

PART II.

OBSERVATIONS ON THE CHEMISTRY OF THE ADRENAL CORTEX. THE ISOLATION OF A HEXURONIC ACID.

1. *Evidence of a strongly reducing agent in the adrenal cortex.*

It has been shown in the first part of this paper that in the plant the peroxidase is connected with a strongly reducing substance, or a group of such substances. This factor of the peroxidase system could be recognised by its high reducing power.

To see whether the adrenal cortex contains any such reducing agent the gland was cut with a knife into halves which were immersed in a 0·4 % $AgNO_3$ solution. In a few minutes the cortical part was seen to darken through the deposition of reduced metallic silver. In a quarter of an hour the cut surface of the cortex turned completely black, while the medulla remained practically uncoloured (see Plate VI, Fig. 1). The black coloration ends towards the medulla with a sharp margin, which corresponds to the anatomical margin between medulla and cortex. Like the medulla, other organs such as brain, liver and muscle remain practically uncoloured.

If in place of the silver a reagent is used by which adrenaline can be detected, the reverse picture is obtained. If for instance the gland is immersed in 5 % potassium iodate solution, containing 30 % acetic acid, within 30 minutes the medulla turns dark violet, while the cortex remains uncoloured.

The adrenal cortex, therefore, contains some specific, highly reducing agent, which can be no artefact, since its action is produced in the almost fresh material.

If the cortical part is isolated from the medulla and a methyl alcoholic solution prepared from the former, the extract will be found to reduce iodine and phosphotungstic acid in higher degree than can be accounted for by the glutathione present and in much higher degree than the extracts of any other organ. Neutral $AgNO_3$ is also reduced at once.

That this reducing agent is a specific constituent of the interrenal system can clearly be demonstrated in elasmobranch fishes (freshly killed *Scyllium canicula*). If the interrenal body is immersed together with another tissue (liver) in 0·2 % $AgNO_3$ solution, in a quarter of an hour the interrenal gland is found to be dark, while the liver is unchanged (if not exposed to light).

2. *Isolation of the reducing substance from the adrenal cortex.*

Frozen ox-glands were used which were cut out from the freshly killed animal at the abattoir in London, where they were cooled to − 6°. The glands were transferred to Cambridge in a frozen condition, and kept there at − 17°. No material older than a few weeks was used, since the reducing substance disappears completely in a few months even at this low temperature. The

disappearance is probably due to oxidation, since it begins in the peripheral parts and proceeds towards the centre of the gland.

In the course of purification the reduction of iodine was used as the leading reaction. At a weakly acid reaction the reducing agent can be titrated with a sharp end-point with 0·01 *N* iodine. As soon as the glutathione had been removed, the iodine reduction gave a quantitative measure of the reducing agent, which, compared with the weight of the dry residue, gave a measure of the purity.

The following properties of the substance were of use in the final method of isolation.

Solubility. In all preliminary experiments the substance behaved as an acid without basic groups.

The free acid is exceedingly soluble in water, freely soluble in methyl alcohol, less readily soluble in ethyl alcohol and acetone, slightly soluble in butyl or amyl alcohol, insoluble in ether or light petroleum. From its alcoholic solution—if not in very high concentration—it is not precipitated by ether, but is precipitated to a great extent by light petroleum. It is precipitated nearly completely from acetone by light petroleum.

Sodium, barium and ammonium salts. The sodium and ammonium salts are freely soluble in methyl alcohol, less readily soluble in ethyl alcohol, slightly soluble in acetone, insoluble in ether. Both salts are precipitated from alcoholic solution by ether. Still smaller is the solubility of the barium salt in the above solvents. All three salts are soluble in water.

Lead salts. The substance forms two different salts with lead. With neutral lead acetate it forms a compound, soluble in water, insoluble in alcohol. It is necessary to employ an excess of lead to obtain a quantitative precipitation.

The substance is precipitated by basic lead acetate equally from water and alcohol.

Stability. In absence of oxygen the substance is stable in water, is not destroyed by short boiling, and can be treated with dilute mineral acid. In anhydrous solvents, such as alcohol or acetone, it is extremely sensitive to mineral acid, and resinifies readily under their influence. Even strong organic acids, such as picric acid, effect its resinification.

The substance is highly autoxidisable. It is oxidised irreversibly by molecular oxygen. The oxidation is greatly catalysed by OH ions, and heavy metals, such as copper. It is inhibited by cyanide.

The substance is not fermented by yeast.

Crystallisation. The free substance does not crystallise under any conditions, but forms an anhydride, which readily crystallises, and can be easily recrystallised. Dissolved in water this readily adds water to form the acid.

Method of isolation. For the extraction of every kg. of glands three litres of methyl alcohol are used. To every litre of the alcohol 0·1 cc. 10 % NaCN is added. CO_2 is led through the alcohol to remove oxygen, and the alcohol is cooled on ice.

The glands are minced in a frozen condition to a fine pulp which is immediately mixed with alcohol. The suspension is allowed to stand for half an hour, being stirred frequently by a strong current of CO_2. Then with vigorous stirring a 50 % solution of barium acetate is added sufficient to make the final concentration 0·5 %.

After standing on ice for another half hour and with occasional stirring, the suspension is rapidly filtered through muslin, the residue being pressed out in a hand press. The fluid is rapidly filtered through paper pulp and the clear filtrate is transferred to bottles. These are kept on ice, the air in the bottles being replaced by CO_2.

A hot saturated solution of lead acetate is added, sufficient to bring the final concentration to 5 %. The fluid is allowed to stand on ice for another hour and is then filtered on a Büchner funnel, the paper in which has been covered with a thin sheet of fine asbestos. The precipitate is washed with methyl alcohol[1].

The precipitate is suspended in as small a volume of water as possible and decomposed by sulphuric acid. The acid is added as 20 % solution in small quantities with stirring and ice-cooling, excess being avoided. The lead sulphate is filtered off. If the filtrate colours thymol blue, sodium bicarbonate is added till the coloration disappears, then the fluid is reduced at 20–30° *in vacuo* to small volume and dried in a vacuum desiccator over sulphuric acid. To absorb acid vapour filter-paper soaked in strong NaOH solution is placed in the desiccator.

The dry residue is extracted with a small quantity of absolute methyl alcohol. To the methyl alcohol five times its volume of freshly distilled water-free ether is added. The precipitate is separated and added to the insoluble residue, which is then extracted again with a small quantity of methyl alcohol, to which again ether is added. The clear alcohol-ether fluids are united. In this way the residue corresponding to 1 kg. of the gland is extracted three times with 10 cc. of methyl alcohol.

The alcohol-ether is cooled in ice, and dry ammonia gas is passed in until the fluid contains an excess of ammonia. Then the fluid is filtered and the precipitate dissolved in a small quantity of water, to which a sufficient quantity of acetic acid has been added to make the final reaction acid. For 1 kg. of the gland 50 cc. of dilute acetic acid solution are used.

After the precipitate has been dissolved the fluid is neutralised by the addition of ammonia, and then 20 % of lead acetate is added in excess in the form of a hot saturated solution. If any precipitate is formed, this is separated on the centrifuge, and then to every 50 cc. of the fluid 150 cc. 96 % ethyl alcohol is added. The fluid is cooled for an hour on ice, and then filtered.

The lead precipitate is dried over sulphuric acid *in vacuo.*

The dry precipitate is powdered and suspended in water-free, freshly

[1] If more convenient, this precipitate can be placed over sulphuric acid in the vacuum desiccator, and kept there for a few days.

distilled acetone, for every kg. of the gland about 100 cc. acetone being used. Then H_2S is passed through until the whole precipitate is decomposed, the acetone is then evaporated at low temperature *in vacuo* to a very small volume. To this an equal amount of ether and an excess of light petroleum are added, which precipitate an oily material, and keep most of the acetic acid in solution. The oily precipitate is transferred to an Erlenmeyer flask (the distilling flask being washed out with a few cc. of methyl alcohol). Here the syrup is dried again over sulphuric acid *in vacuo*, NaOH paper being placed in the desiccator.

The solid residue is extracted repeatedly with small volumes (5 cc.) of water-free acetone, which is then evaporated *in vacuo* at low temperature. The residue is dissolved in a very small volume (2–3 cc.) of methyl alcohol. To this 15 cc. of water-free ether are added. If an amorphous precipitate forms, it is separated and extracted with acetone and the acetone treated as above.

To the clear alcohol-ether an excess of light petroleum is added (10 times the volume of the fluid), and the mixture placed in the ice-chest.

The next morning a crystalline precipitate is found on the bottom of the flask with some oily material. The crystals are freed from the oil by washing with a small amount of ice-cooled acetone. Water-free ether is used to wash the crystals out of the flask and remove the last trace of acetone.

The main impurity of the preparation in the last stages are resinification products of the substance itself. These are less soluble in acetone and alcohol-ether than the unchanged substance.

The yield of the preparations, working with quantities of 1–3 kg. of the gland, is 300 mg. per kg. This corresponds approximately to half the total amount present in the gland.

The dried crystals are stable *in vacuo*, and decompose slowly in the air.

3. *The chemical nature of the reducing substance.*

The crystalline preparation can be recrystallised most conveniently by dissolving the crystals in a small volume of absolute methyl alcohol, and gradually adding ether and light petroleum.

In presence of water the substance does not crystallise but forms a sticky syrup. This syrup, however, on standing *in vacuo* over sulphuric acid slowly loses water and solidifies to a crystalline mass.

The substance is polymorphic. The primary crystalline preparation usually consists of small plates, which combine to more complicated figures (Plate VI, Fig. 2 *a*). Recrystallised from acetone with ether the same crystals, or needles, may be obtained (Fig. 2 *b*). Different structures are obtained by evaporating a methyl alcoholic solution on an object slide (Fig. 2 *c*), a watery solution in a thin layer (Fig. 2 *d*), or in a larger quantity (Fig. 2 *e*).

The preparations of the substance show a fairly sharp melting-point, ranging between 175 and 189°. A few degrees below the M.P. the substance turns first yellow and then brown, and after melting forms a brown resin.

The M.P. of my successive preparations were: 175, 183, 175, 186, 186, 189, 185, 182°.

Molecular weight. The minimum molecular weight could be determined by two different methods, by acidimetric and by iodometric titration.

4·993 mg. of the substance were dissolved in 3 cc. of water. Titration in presence of phenolphthalein with 0·0216 *N* NaOH required 1·51 cc. Thus the equivalent weight = 178.

After the titration had been finished, one drop each of 33 % acetic acid and starch solution was added, and the mixture was titrated with 0·01 *N* iodine, requiring 5·66 cc. corresponding to an equivalent weight of 88·7.

The experiment shows that the minimum molecular weight given by the acidimetric titration is 178, and that for each carboxyl group two atoms of iodine are reduced.

The molecular weight was estimated by the method of Barger.

9 mg. of the substance were dissolved in 0·5 cc. of water. This was sealed in capillaries with 0·4, 0·2, 0·1 and 0·05 *M* urea solutions. The tubes were left for 24 hours at 37°. In the tubes 1 and 2 the urea solution was hypertonic. No movement of the meniscus occurred in tube 3; in tube 4 the urea was hypotonic; whence M.W. = 178 (± 2 %).

Optical activity. 30 mg. of the substance were dissolved in 1·5 cc. of water: temp. 21°. The rotation was + 0·48° in a 1 dm. tube. This corresponds to $[\alpha]_D^{21^\circ} = + 24°$[1]. The substance showed no mutarotation. (Observed for a week.)

Elementary analysis. The elementary analysis was made by "Feinchemie, Tübingen." In the qualitative analysis the substance shows no N, Cl, P, S, or OMe.

4·600 mg. gave 6·865 mg. CO_2 and 1·97 mg. H_2O.

	Found %	Calc. for $C_6H_8O_6$ (M.W. 176) %
C	40·7	40·9
H	4·7	4·5
O	54·6	54·6

Chemical reactions. Heated in dry condition the substance resinifies, giving a strong smell of caramel.

Fehling's solution, alkaline or neutral silver and permanganate are readily reduced at room temperature.

The Molisch reaction is strongly positive with a purple colour.

The orcinol test (with Fe) is also strongly positive.

The substance gives thus all the typical carbohydrate reactions tested.

Constitution. The above data make it clear, that the reducing substance is a highly reactive carbohydrate derivative, isomeric with glycuronic acid,

[1] The accuracy of the reading of optical activity lies within 10 %.

which, by losing water, goes over reversibly into the crystalline lactone anhydride, corresponding to the formula $C_6H_8O_6$.

No attempt was made in the present research to elucidate the steric configuration of the molecule. In the latter part of the paper some arguments are given in favour of the view that the substance is a hexuronic acid derived from fructose.

The substance seems not to be identical with any of the glycuronic acid isomers hitherto described.

The high reactivity excludes identity with glycuronic acid. The solubility of the calcium salt and the different optical activity excludes the identity with the acid investigated by Bertrand [1904], Boutron [1886], Ruff [1899] and Kiliani [1922]. The high reactivity and the different melting-point and optical activity exclude the identity with the galactosuric acid, isolated from plants by Ehrlich and Sommerfeld [1925].

Since the exact constitution of the reducing substance is unknown, I propose to refer to it as a hexuronic acid.

A few words may be said about the specificity of this substance for the adrenal cortex.

As has been shown above, the substance is specific for the cortex in as far as no other animal organ contains it in amounts comparable to those present in the interrenal tissue. But whether other tissues contain it in small quantities, or whether the substance is absent from other organs altogether, cannot be stated at present. The extracts of all organs have some reducing action, 8–20 times smaller than that of the adrenal cortex. How far this reduction is due to hexuronic acid, or to other substances, cannot be stated, since the quantities possibly present are too small for isolation, which is the only reliable way of identification. As will be shown in a later part of this paper, the hexuronic acid is extremely active as a catalyst in certain systems of oxido-reduction. Concentrations of 1 : 0·000025 are sufficient to show a distinct biological activity, so that this substance may play an important rôle in the oxidation mechanism of all animal tissues.

PART III.

ISOLATION OF THE HEXURONIC ACID FROM PLANTS AND SOME OBSERVATIONS ON THE CHEMISTRY OF THE REDUCING FACTOR.

1. *The isolation of the hexuronic acid from plants.*

After the study of the reducing factor had led to the isolation of a hexuronic acid from the adrenal cortex, the question arose whether this acid actually occurs among the reducing substances of the plant. This question could be answered only by the isolation of the acid.

Before the method used for the adrenal could be applied, the water of the plant juice had to be eliminated, and the bulk of accompanying substances reduced. A great number of accompanying impurities could be eliminated by precipitation with barium and alcohol. The water could be eliminated by vacuum distillation. The most effective step in the purification was the precipitation of the acid by lead from its alcoholic solution at slightly acid reaction. This precipitation is however inhibited in the crude juice, and it was necessary to precipitate the substance first with basic lead to eliminate the inhibitory factor.

After this preliminary treatment the original adrenal method was adopted almost unchanged, and led to the isolation of a substance identical with hexuronic acid. This substance, which forms in the animal a specific constituent of the adrenal cortex, seems to be widely distributed in the vegetable kingdom, forming an essential constituent of the reducing substances, apparently connected with the function of the peroxidase system.

As starting material for the isolation, orange juice and the watery extract of cabbages were chosen.

2. *Isolation of the hexuronic acid from oranges.*

Method. The ripe fruit is cut in two, and squeezed out on a lemon squeezer. The juice is filtered through muslin. To every litre 0·1 cc. of 10 % sodium cyanide is added. By addition of ammonia the fluid is then brought to p_H 6·2, and 5 % barium acetate is added in the form of a hot saturated solution. The fluid is left in full closed bottles overnight in the ice-chest and filtered the next morning through paper pulp. The filtrate is concentrated *in vacuo* at 25° to one-fifth of its volume. The concentrated fluid is cooled in ice and filtered from the newly formed precipitate. Again sodium cyanide (0·1 cc. 10 % to 1 litre) and then hot saturated lead acetate are added, sufficient to make up the total concentration to 10 %. The fluid is cooled in ice, then with vigorous stirring ammonia is added until the fluid just colours bromothymol blue. The fluid is quickly filtered, the precipitate washed with water and suspended in the smallest possible volume of water and decomposed with sulphuric acid, excess being avoided. After the lead sulphate is removed by

filtration, the p_H is adjusted to 6. If necessary, the volume of the fluid is reduced by vacuum distillation to one-twentieth of the original bulk. Three times its volume of 96 % alcohol is then added, the precipitate, if formed, separated and the fluid mixed with excess of hot saturated lead acetate. The fluid is cooled on ice for an hour and filtered. The precipitate is treated further by the method described in the first part of this paper.

The crystalline precipitate obtained consists of small irregular crystals. Recrystallised these show the same polymorphism as the substance isolated from the adrenal gland. The M.P. in the different preparations was 182, 178 and 175°.

The acidimetric titration gives an equivalent weight of 180 (± 2 %). The more accurate iodometric titration gives an equivalent weight of 89. Thus for each acid group two atoms of iodine are reduced and the minimum M.W. given by the titration is 178 g.

The estimation by Barger's method gives M.W. 178.

The substance has $[\alpha]_D^{20°} + 24°$, and displays no mutarotation.

Analysis (by "Feinchemie Tübingen") gives theoretical values for $C_6H_8O_6$.

	Found %	Calc. for $C_6H_8O_6$ %
C	40·9	40·9
H	4·5	4·5
O	54·6	54·6

All chemical reactions were identical with those found with the acid from the adrenal cortex.

These data sufficiently establish the identity of the substances isolated from the adrenal cortex and from orange juice. Identical substances were obtained from the orange juice in three subsequent preparations. Working with 1·5–3 litres of juice the yield is about 100 mg. per litre.

Isolation of the hexuronic acid from cabbages.

The isolation of the acid from cabbages is made still more difficult by the accompanying substances.

The fresh leaves are immersed in 1 % acetic acid, for each kg. of the plant 1 litre being used. Then the fluid is heated to 80°, cooled, and pressed out in a mechanical press. To every litre 0·1 cc. 10 % NaCN is added. Sufficient barium acetate is then dissolved in the fluid to make it up to 1 %. Then a hot saturated solution of lead acetate is added, sufficient to make the final concentration 7·5 %. The precipitate is separated and the fluid cooled in ice, made alkaline with ammonia (to bromothymol blue) and filtered. The precipitate is treated in the same way as that from the oranges.

From 5 litres of juice 250 mg. crystalline matter were obtained, of the same crystal structure as the substance from oranges; M.P. 188°. In all reactions this substance behaves in the same manner as that isolated from oranges.

The method does not seem to work on a larger scale with this material. Two attempts to isolate the acid from 300 kg. and 50 kg. were fruitless.

3. *The chemistry of the reducing factor.*

It has been shown in the first part of this paper that 100 cc. of orange juice reduce about 60 cc. of 0·01 *N* iodine. This would correspond to about 50 mg. hexuronic acid anhydride and about 10 mg. of this substance can actually be isolated in crystals, corresponding to 11 cc. of 0·01 *N* iodine. Since the preparation is accompanied by great losses it is clear that this compound must form an essential part of the "reducing factor." But the reagents employed for the demonstration of the R.F. are not specific, so we can hardly expect that the whole R.F. should be constituted by the hexuronic acid. Phosphotungstic acid and silver are reduced by most phenolic substances, and iodine is reduced by many phenols. Thus we can expect that the phenols present would constitute a part of the R.F. if the above reagents are employed. In fact there is present in orange juice, as in cabbage juice, a phenolic substance in rather high concentration, which reduces all three of the above reagents, so that about half of the R.F. must be composed of this phenolic substance.

The reducing properties of plant juice have repeatedly attracted attention, specially from students of vitamin C. Bezssonoff [1921] has applied Folin's phosphomolybdic acid reagent. The reducing substances of lemon juice have been made the object of a thorough study by Zilva [1927, 1928], who established interesting relations between vitamin C and the reducing properties of the plant juice. The main reagent employed by Zilva [1928] was phenol-indophenol. Indophenol blue is readily reduced by the hexuronic acid, so that it is probable that it was this substance which has been studied by Zilva. Phenols are less likely to reduce this indicator.

PART IV.

OBSERVATIONS ON THE OXIDATION AND REDUCTION OF THE HEXURONIC ACID.

1. *The reduction potential of the hexuronic acid.*

A 1 % solution of the acid was neutralised with 0·1 *N* NaOH, then phosphate buffer (p_H 7) was added and the mixture diluted with water. The final concentration of the acid was 0·2 %, that of the buffer 0·01 *M*.

This solution has been added to the series of Clark indicators. Nos. 1, 2 and 3 were not reduced. No. 4 was reduced fairly rapidly. No. 8 was readily reduced. Methylene blue was reduced slowly to completion.

The preliminary measurement of the potential with the gold electrode showed that the substance has a definite reduction potential and decreases the E_H of the pure buffer solution from + 240 mv. to + 90 mv., corresponding to an r_H of 17·14.

The hexuronic acid shows thus, like glutathione and thyroxine, a distinct and fairly high reduction potential.

I am indebted to Dr M. Dixon for his kind assistance in these measurements.

2. *Oxidation by iodine.*

A small quantity of the acid is dissolved in water; 1 cc. of the solution reduces 1·3 cc. 0·01 *N* iodine.

Three further samples of 1 cc. are taken. To each one drop of starch is added. To the first sample an alcoholic solution of iodine is added, till the first persistent blue colour appears. The excess of iodine is reduced by the addition of a trace of the acid. Then to each of the samples thymol blue is added. The first sample which has been oxidised is acid, the others alkaline to thymol blue. Then to the two samples which have not been oxidised 0·01 *N* HCl is added until they reach the colour of the first sample, 1·26 cc. and 1·23 cc. being required.

Thus for each atom of iodine added 1 mol. of HI was formed, the oxidation of hexuronic acid consisting therefore in the removal of two H atoms.

3. *Oxidation by molecular oxygen.*

The solution used in the potential measurement is placed in the Barcroft apparatus and shaken at room temperature. 4 mm.^{3} O_2 are taken up per minute.

After 10 minutes the apparatus is opened and to the fluid 1 cc. of 0·01 *M* neutralised KCN added. No further oxygen is taken up on shaking at room temperature.

This experiment shows that, corresponding with its reducing potential, the substance is fairly autoxidisable. As the autoxidation is inhibited by cyanide, it must be a heavy metal catalysis.

The following experiment was made on the nature of the heavy metal.

To a 0·01 *N* (iodometric) solution of the hexuronic acid a double volume of 0·1 *M* phosphate buffer of p_H 7 was added. The oxygen uptake of 3 cc. of this fluid was measured in the Barcroft apparatus, 3 cc. of water being used in the compensating flask. Then the oxygen uptake of 3 cc. of the same fluid was measured in presence of 0·1 cc. 0·005 *N* $FeCl_3$, $CuCl_2$ and manganese acetate. Without metal, or in the presence of Fe or Mn, the acid showed no measurable oxygen uptake. In presence of copper the fluid showed an intense uptake of oxygen (20 mm.^{3} per minute). In 15 minutes the oxidation of the acid is complete. The autoxidation of the hexuronic acid is thus greatly catalysed by copper, but it is not catalysed, or catalysed in much smaller degree, by iron and manganese.

Since the acid is readily oxidised by trivalent iron, the inactivity of this substance as a catalyst gives evidence against those theories which explain the catalytic action of iron by its reduction by the autoxidisable substance and its subsequent autoxidation.

The oxidation of the acid by H_2O_2 is also greatly catalysed by copper.

4. *The chemical nature of the oxidation products.*

If the hexuronic acid is oxidised by iodine or silver, it can be reduced again quantitatively by reducing agents such as H_2S. The two atoms of H which are given off on oxidation can thus be again replaced.

If Fehling's solution is added to the unoxidised acid, the reagent is readily reduced at room temperature. After a while the reduction stops, but proceeds again on boiling. But if the acid is oxidised first by iodine or silver, Fehling's solution is not reduced at room temperature. If however the oxidised acid be heated with Fehling's solution the reagents are readily reduced. The Tollens silver reagent shows an analogous behaviour. This shows that the aldehyde or ketone function of the acid is not involved when the substance is oxidised at room temperature by iodine, silver or Fehling's solution. It is thus not this function to which the high reducing power is due.

If the substance is oxidised by molecular oxygen at neutral reaction, copper being used as catalyst, the oxidation product which is unable to reduce iodine, neutral silver, or Fehling's solution at room temperature still reduces the latter reagent on boiling. Thus the aldehyde or ketone function is not involved in the autoxidation. But if the acid is oxidised by oxygen, the oxidation product is irreversible, and cannot be reduced again by H_2S. If the fluid is titrated with alkali, it is found that new acid equivalents are formed on autoxidation[1]. The oxidation of the two labile hydrogens leads thus to the formation of carboxyl groups, which makes it very probable, that it is a terminal carbon atom which carries the two labile hydrogen atoms. The lability of these hydrogen atoms makes it probable that their carbon atom is next to the carbonyl group. This would correspond to the structure of a fructuronic acid, which can be expected to have a γ-lactone oxygen since it shows no mutarotation.

5. *The oxidation by peroxidase and peroxide.*

It has been shown in the first part of this paper that in the plant juice the hexuronic acid is readily oxidised by peroxide and peroxidase.

After the substance had been isolated, purified peroxidase and peroxide were added to its solution, in order to study this oxidation. The experiment gave the unexpected result that purified peroxidase had no effect on solutions of the crystalline compound. If to its solution some plant juice (cabbage or orange) was added, together with the peroxide and peroxidase, it was oxidised at a high rate. This made it clear that the peroxidase has no direct action on the acid, and the rapid oxidation in the plant juice is due to the presence of catalytic substances.

[1] Corresponding with the high rate of autoxidation the new carboxyl groups are formed rapidly. Every molecule of hexuronic acid forms one new carboxyl group. When these new carboxyl groups have been established, the substance goes on forming new acid groups, but at a very low rate. This further formation of acid groups is probably due to the breaking up of the molecule.

Much time was given to the study of the nature and action of these catalysts. The details of this extended study will not be given here to avoid a further extension of the present paper. It was found that the catalysts of this reaction are aromatic substances which play the rôle of a catalytic hydrogen carrier between the acid and the peroxide or peroxidase. The phenolic substances are in this reaction oxidised to quinones which in their turn oxidise the labile hydrogen of the acid, being themselves reduced again to their original phenolic form. Thus any phenol which is oxidised by peroxide and peroxidase will be able to catalyse the reaction between peroxide and peroxidase on the one side, and hexuronic acid on the other.

In the plant, therefore, there are two catalytic hydrogen carriers between the peroxidase and oxidation systems, in which the hydrogen of the foodstuffs is activated. The scheme of oxidation is thus the following:

H_2O_2 + peroxidase → phenol → hexuronic acid → other oxidation systems.

6. *The oxidation in coupled systems.*

What the source of the peroxide is in the living tissue and where it is generated, are as yet unknown. Different oxidation systems are known, which are normal cell constituents and which lead to the formation of peroxide. Wieland and Sutter [1928] have shown that the thermostable oxidase, acting on quinol produces hydrogen peroxide in equivalent amount to the oxygen adsorbed. Thurlow [1925] has shown that the oxidation of acetaldehyde by the Schardinger enzyme is connected with the formation of peroxide.

We may expect that the H_2O_2 formed in any of these or other systems can be used for the oxidation of the hexuronic acid, and it seemed worth while to demonstrate that the H_2O_2 formed in such an oxidation can actually be used for the oxidation of this component.

The Schardinger enzyme was used as material for this experiment. It might be expected that in the presence of O_2 and acetaldehyde the enzyme would generate H_2O_2, which, in presence of peroxidase, would act on an aromatic substance which in its turn would oxidise the compound. It would be expected that the oxidation of the phenol would reveal itself in the formation of the coloured quinone, and that in presence of hexuronic acid the solution would remain uncoloured, owing to the reduction of the quinone by the acid.

To 1000 cc. of fresh milk 10 cc. rennin were added, the milk was warmed to 37°, and the whey separated by filtration through muslin. To the whey an equal volume of saturated ammonium sulphate was added, the fluid filtered and the precipitate washed with half saturated ammonium sulphate. The precipitate was dissolved in 150 cc. water, precipitated with 150 cc. ammonium sulphate and washed as above. The precipitate was extracted with ether, dissolved in 150 cc. of water, once more precipitated with ammonium sulphate, and finally dissolved in 300 cc. 0·1 *M* phosphate buffer of p_H 7.

2 cc. of this solution *in vacuo* in presence of 0·02 cc. of acetaldehyde

reduces 0·1 cc. 0·1 % methylene blue in 90 seconds at room temperature and in 15 seconds at 37°.

On addition of peroxide and peroxidase the fluid slowly develops a faint blue colour, showing that it contains but little peroxidase.

In all experiments 2 cc. enzyme, 0·02 cc. acetaldehyde, 0·2 cc. 0·1 % adrenaline and 0·1 cc. 1 % peroxidase (turnip) were used.

2 cc. of the enzyme solution were placed in a test-tube, aldehyde, peroxidase and adrenaline added, the tube was immersed in water at 37° and aerated. Within one minute a strong red colour indicated that the adrenaline had been partly oxidised to quinone. It is easy to prove that the adrenaline has in fact been oxidised by the peroxide formed in the oxidation of the aldehyde. If the aldehyde or the Schardinger enzyme are omitted, or if the Schardinger enzyme is inactivated by boiling, no coloration occurs. If the peroxidase is left out, only a very faint colour occurs (owing to the slight amount of milk-peroxidase present). Also in presence of cyanide the coloration remains very faint. The experiment gives very clear results and gives a very striking and simple demonstration of the formation of H_2O_2 in biological oxidation.

If the hexuronic acid is added to the same system, the mixture remains uncoloured as long as there is unoxidised acid present. As soon as the acid is used up, the red colour appears. Thus the appearance of the red colour coincides with the disappearance of reduced hexuronic acid, judged by the phosphotungstic reaction. If to the above system 0·05 mg. of hexuronic acid is added, the fluid remains uncoloured for one minute. With 0·1 mg. it remains uncoloured for two minutes, with 0·2 mg. for 4 minutes, with 0·4 mg. no colour appears at all during the 9 minutes of observation.

Thus even a concentration of 0·0025 % of the acid is sufficient to prevent the formation of the pigment, *i.e.* to reduce the quinone formed, as long as the acid is present in reduced condition. Since tissues, as has been shown in the first part and will be shown again later, energetically reduce oxidised hexuronic acid, it can be expected that in the cell the oxidised acid will be reduced again, and in this way be maintained in reduced condition. In this case the concentration of 0·0025 % will be sufficient to show a distinct biological activity.

This experiment deserves perhaps a special consideration since it shows that, in a system composed of normal cell constituents, the formation of pigment can be prevented by a specific constituent of the adrenal cortex. It may be remembered that one of the best known symptoms of a deficiency of the interrenal system is the formation of iron-free pigment.

6. *Oxidation by the indophenoloxidase.*

As is known from the work of Battelli and Stern [1912] washed muscle contains a very active enzyme, capable of oxidising *p*-phenylenediamine at a high rate. The recent experiments of Keilin [1929] make it probable that this enzyme plays a very important part in tissue respiration.

It was found that this enzyme is unable to oxidise the hexuronic acid. If muscle is washed and shaken in the Barcroft apparatus with a solution of

the acid, no oxygen uptake is observed and the hexuronic acid is found unoxidised at the end of the experiment.

It may also be noted that the reduction of methylene blue by the hexuronic acid is not catalysed by muscle. Thus the hydrogen of the acid is not activated by tissues, which makes it probable that the substance cannot be oxidised definitely and used as foodstuff by the animal cell.

7. *Oxidation by haematin compounds.*

The hexuronic acid reduces haematin compounds, but the rate of reduction is too slow to indicate that the acid is actually connected in its function with these respiratory pigments.

Experiments were made with cathaemoglobin and cytochrome. Cathaemoglobin is reduced by small amounts of hexuronic acid *in vacuo* overnight. No effect can be observed in a short experiment.

A solution of cytochrome C is reduced in a few minutes by small quantities of the acid. The cytochrome of washed muscle is reduced in about 5 minutes. The cytochrome of the washed muscle is reduced by hexuronic acid about 30 times more slowly than by succinate (which is acted on by the succinodehydrase).

The oxidation of the hexuronic acid by hydrogen peroxide is greatly catalysed by haematin compounds (cathaemoglobin). The concentration of hydrogen peroxide which is necessary to effect a rapid oxidation of the acid is much too high to assume that the haematins would act *in vivo* as peroxidases on hexuronic acid.

I am indebted to Dr D. Keilin for his kind assistance in these experiments.

8. *The reduction of oxidised hexuronic acid by animal tissues.*

If an animal tissue, such as kidney, liver or muscle, is minced and suspended in a little buffer solution, and oxidised hexuronic acid is then added and the suspension incubated for a short time, for instance 15 seconds, the hexuronic acid will be found to be partly present again in reduced form at the end of the incubation.

Exp. The kidneys (1·8 g.) of a freshly killed rat are cut into pieces, and then minced in a mortar with a little sand. 2 cc. 0·2 *M* phosphate buffer of p_{H} 7 is added, and the suspension divided into two equal parts. To one part 0·5 cc. water is added, to the other 0·5 cc. oxidised 0·01 *M* hexuronic acid (oxidised with iodine). The suspensions are incubated for 15 minutes in a water-bath. Then equal parts of 6 % trichloroacetic acid are added to both. The samples are centrifuged. The fluids are tested with phosphotungstic acid for reduced hexuronic acid and with sodium nitroprusside for glutathione. A portion of both samples is neutralised with $NaHCO_3$, and silver nitrate added. The fluid with hexuronic acid gives a weaker —SH reaction with nitroprusside, but a much stronger reaction with phosphotungstic acid, and reduces silver nitrate.

Two oxidising systems are known in animal tissues which might be expected to reduce oxidised hexuronic acid: the Thunberg-Wieland hydrogen-activating system, and the Hopkins glutathione system.

9. *The Thunberg-Wieland system.*

Two main types of enzymes are known in this system: the type of the Schardinger enzyme system and the type of the succinoxidase. The former are soluble, and consist only of a hydrogen activator, the latter are insoluble and consist of a hydrogen activator and an oxygen activator [Fleisch, 1924; Szent-Györgyi, 1927, 3].

The Schardinger enzyme. This enzyme appeared to be unable to reduce oxidised hexuronic acid. The following experiment may be noted.

Exp. 0·2 % hexuronic acid is oxidised with 0·1 *N* iodine-KI solution. To 4 cc. of the above enzyme solution 0·04 cc. acetaldehyde and 0·4 cc. of the oxidised acid are added. The phosphotungstic acid reaction after 10 minutes' incubation is negative.

The *succinodehydrase* was also found to be inactive.

Exp. Ox diaphragm muscle was minced and washed three times with 20 times its own volume of distilled water, shaking it for 15 minutes. The muscle was filtered through muslin and squeezed out.

2 g. of this muscle in 10 cc. buffer (0·2 *M* phosphate of p_H 7) in presence of 0·1 % succinate reduces 0·4 mg. methylene blue in 9 minutes.

1 g. of this muscle is suspended in 2 cc. phosphate, then 0·25 cc. 2 % sodium succinate and 0·5 cc. 0·2 % oxidised hexuronic acid are added. The suspension is incubated in evacuated Thunberg tubes for 35 minutes. In control experiments the succinate, the muscle and the hexuronic acid are replaced by water.

After the incubation the tubes are cooled in ice, opened, and the liquid tested with phosphotungstic acid.

The phosphotungstic acid reaction is negative in the tubes containing no muscle, positive in the tubes with muscle. Thus in the presence of muscle a small part of the oxidised hexuronic acid has been reduced. But, since the reaction is equally strong in tubes with and without succinic acid, the reduction is not due to the succinodehydrase.

The reduction of methylene blue by muscle and succinate is not inhibited by oxidised hexuronic acid. This substance therefore does not inhibit the dehydrase.

10. *Reduction by the Hopkins glutathione system.*

Having found that the Thunberg-Wieland dehydrases do not reduce the substance it seemed probable that it is the Hopkins system to which the reduction is due. This system consists of glutathione, fixed SH-groups and a hitherto unknown factor, reducing the glutathione, either directly or through the fixed SH-groups.

Reduction by fixed SH-groups. It has been shown in the last experiment that the washed muscle reduces oxidised hexuronic acid. The experiment was completed by carrying out the nitroprusside reaction with the muscle after its incubation with oxidised hexuronic acid. The experiment showed that the

reaction for fixed SH-groups became much weaker if the muscle was incubated with the oxidised acid. This shows that the acid is reduced by fixed SH-groups.

Reduction by glutathione. If oxidised hexuronic acid is incubated at neutral reaction with reduced glutathione, the former becomes reduced, the latter oxidised.

Exp. A 0·02 *N* (iodometric) solution of reduced glutathione and a 0·06 *N* solution of hexuronic acid are prepared. The latter is oxidised with a strong alcoholic solution of iodine. Both solutions are neutralised with 0·1 *N* NaOH. To both one-tenth part of 0·2 *M* phosphate buffer (p_H 7) is added. Equal parts of both fluids are mixed and incubated. The quantity of SH is roughly measured by the intensity of the nitroprusside reaction. The experiment shows that in $2\frac{1}{2}$ minutes about one-third, in 5 minutes about two-fifths, and in 10 minutes about half of the glutathione is oxidised. (The phosphotungstic reaction cannot be used in this experiment, since reduced glutathione also reduces phosphotungstic acid, though in much smaller degree than the hexuronic acid.) After incubation *in vacuo* for 90 minutes the —SH reaction reveals only a trace of reduced —SH, showing that the oxidation of —SH is practically complete.

In control experiments, made without phosphate buffer, parallel to the disappearance of the nitroprusside reaction, the silver reduction, which is absent at the beginning, becomes gradually stronger.

The —SH reaction of neutral glutathione does not change under identical conditions, if no oxidised hexuronic acid is present.

The experiment thus shows that glutathione reduces the oxidised acid fairly rapidly. The fixed —SH is also able to reduce the acid. Whether the third factor of the system, which reduces the S—S link, also directly reduces the oxidised acid, or only through the labile and fixed —SH, cannot be stated at present.

Summary.

Part I. If H_2O_2 is added to the juice of a peroxidase plant, the first quantity of the peroxide is used up for the oxidation of a certain substance, or a group of substances, referred to as the "reducing factor" (R.F.). This oxidation of the R.F. is greatly catalysed by the peroxidase. The rate of the oxidation of this factor is very high, and as long as this factor is present no other substances will be oxidised by the peroxidase, and peroxide reactions such as the guaiacum reaction remain negative. The oxidation of the R.F. is a reversible one, and the oxidised R.F. is reduced again by other oxidising systems. The function of this factor is thus that of a catalytic hydrogen carrier between the peroxidase and other oxidising or reducing systems. The R.F. is characterised by its reducing power.

Part II. It is shown that the adrenal cortex contains a relatively large quantity of a highly reducing substance which is specific for the interrenal system. The isolation of the substance is described. Chemical and physical properties of the substance are discussed. It is shown that the substance is a

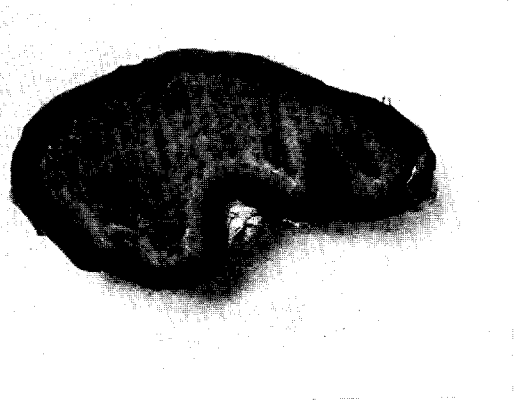

Fig. 1. Cross-section of the adrenal gland after treatment with $AgNO_3$.

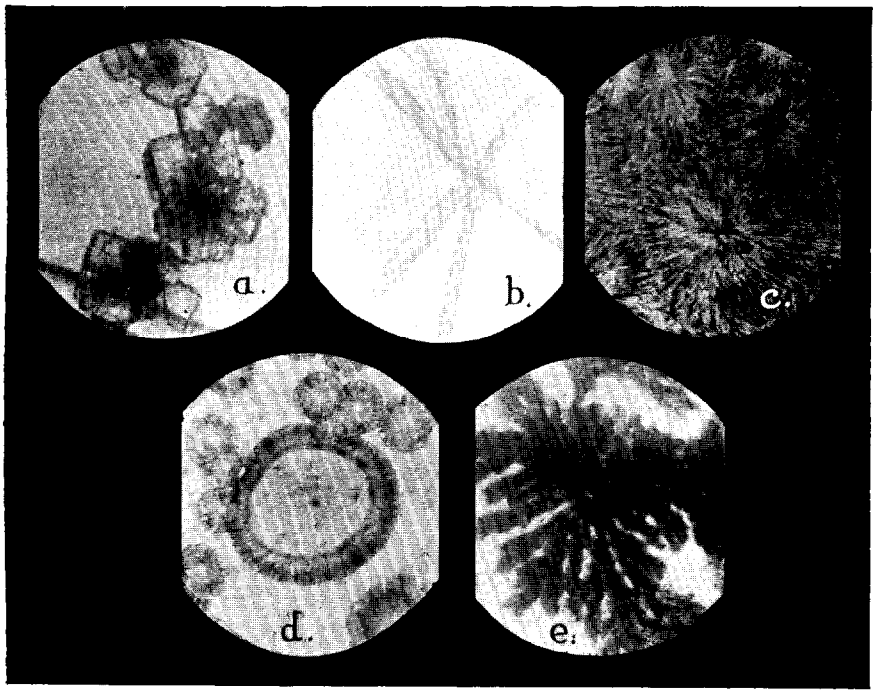

Fig. 2. Crystals of the hexuronic acid formed under different conditions.
Diameter of each field 1·25 mm.

hitherto unknown, highly reactive isomer of glycuronic acid, so that the substance is a hexuronic acid.

Part III. The isolation of the hexuronic acid from oranges and cabbages is described. It is shown that this substance forms an essential part of the reducing factor, the chemistry of which is discussed.

Part IV. It is shown that the hexuronic acid has a definite reducing potential. On oxidation by mild oxidising agents the substance loses two atoms of hydrogen. This oxidation is reversible. The substance is autoxidisable. Molecular oxygen oxidises the substance irreversibly forming new carboxyl groups. This autoxidation is greatly catalysed by copper and inhibited by cyanide. Iron and manganese are relatively inactive as catalysts.

The substance is not oxidised immediately by peroxide and peroxidase. The peroxidase reaction is catalysed in the plant juice by phenols.

It is shown that in presence of peroxidase and a phenol the H_2O_2 generated by other oxidising systems can be used for the oxidation of the hexuronic acid. Pigment formation in experiments *in vitro* can be inhibited by concentrations of 1 : 0·000025 of the acid.

In the absence of phenolic substances the hexuronic acid is not oxidised by the indophenoloxidase of animal tissues.

Haematin compounds (cytochrome) are reduced relatively slowly by the hexuronic acid.

The oxidised hexuronic acid is intensely reduced by animal tissues. The dehydrogenases of the type of the Schardinger enzyme and the succinodehydrase are unable to reduce the oxidised hexuronic acid. The oxidised substance is reduced by the Hopkins glutathione system. Glutathione and fixed —SH strongly reduce the oxidised acid.

It is a great pleasure to express my deepest gratitude to Sir F. G. Hopkins for his extreme kindness and helpfulness in the course of this research.

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